



UNIVERSITI PUTRA MALAYSIA

**ISOLATION AND SEQUENCE ANALYSIS OF CANDIDATE cDNA
INVOLVED IN CAROTENOID BIOSYNTHESIS IN THE FLAVEDO
TISSUE OF PUMMELO (*CITRUS GRANDIS* L. OSBECK)**

UMMI KALTHUM BINTI HANAPI

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By

UMMI KALTHUM BINTI HANAPI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,
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January 2008

Chairman : Associate Professor Dr. Norihan Mohd Saleh, PhD

Faculty : Biotechnology and Biomolecular Sciences

Citrus grandis L. Osbeck (pummelo) was recognized as one of the potential fruit to be commercialized under the goal of the Third National Agricultural Plan's (DPN3, 1998-2010). However, the pale yellowish green colour of *C. grandis* fruit flavedo rendered is unattractive and has less market demand compared to other citrus varieties. Carotenoid biosynthesis has been well studied and recognized to be the main pathway which contributes to the formation of colour in the flavedo of most citrus fruit. However, there is still limited information currently available on carotenoid biosynthesis in green pigmented citrus fruit flavedo. To increase the market demand, locally and overseas, there is a need to modify the colour of *C. grandis* from pale yellowish green to more attractive colour like other citrus. Therefore, the study was initiated to explore the possibility of modifying fruit flavedo colour through the use of molecular techniques. Thus, it is necessary to isolate and sequence the genes involved in carotenoid pathway of green flavedo of citrus fruit. In this study, comparative analysis of the sequences isolated from the

gene of green flavedo will be compared with orange pigmented citrus, other plants and bacteria which contain high carotenoids. The study began with tagging of open pollinated flowers (that showed indication of fruit formation to ensure the right stage of maturation), followed by genomic DNA and total RNA isolation prior to further molecular analyses, such as PCR and RTPCR. The primers used in this study were designed based on the sequence information obtained from the Genbank database. The four genes which are involved in carotenoid biosynthesis and responsible for the orange pigmentation in citrus fruit viz. *phytoene synthase* (*psy*, 0.46 kb), *phytoene desaturase* (*pds*, 1.11 kb), *lycopene β -cyclase* (*lyc*, 0.70 kb) and *β -carotene hydroxylase* (*chx*, 0.23 kb), were isolated via PCR and RTPCR. From sequence analysis, *psy*, *pds*, *lyc* and *chx* showed high similarities with the corresponding genes in citrus varieties, *Prunus armeniaca* and *Capsicum annuum*. A full sequence of *chx* gene was isolated from cDNA library of *C. grandis* fruit flavedo. The 1268 bp of *chx* gene consisted of an open reading frame (ORF) of 271 codons (mass of 65.8 kD) and 170-nucleotide 5' untranslated sequences was isolated. Compared to other organisms, seven histidine residues which were present in *C. grandis chx* were also found to be conserved in *Alcaligenes sp* (accession no. D58422), *Agrobacterium aurianticum* (accession no. D58420), *Erwinia herbicola* (accession no. M87280) and *Pseudomonas putida* (accession no. KT2440 NP_745389). 'Motif 1' and the predicted 'TM helix' regions were also observed in *C. grandis* when compared to *Prochlorococcus marinus* MED4 *CrtL- ϵ* (CAE19092), *Lycopersicon esculentum* *Lcy- ϵ* (O65837), *Tagetes erecta* *Lcy- ϵ* (AAG10428), *Capsicum annuum* *Lcy- β* (Q43415) and *Arabidopsis thaliana* *Lcy- β* (AAA81880). In conclusion, *psy*, *pds*, *lyc* and a full length of *chx* have been successfully isolated the flavedo of *C. grandis*.

The sequences of these genes were found to be highly conserved with the corresponding gene in other plants especially in citrus varieties.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**PEMENCILAN DAN ANALISIS JUJUKAN cDNA TERPILIH YANG
TERLIBAT DALAM BIOSINTESIS KAROTENOID DALAM TISU
FLAVIDO LIMAU BALI (*CITRUS GRANDIS* L. OSBECK)**

Oleh

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Januari 2008

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Citrus grandis L. Osbeck (limau bali) telah dikenalpasti sebagai salah satu daripada buah-buahan yang mempunyai potensi untuk dikomersilkan di bawah Dasar Pertanian Negara Ketiga (DPN3 1998-2010). Walaubagaimanapun, warna hijau kekuningan yang pucat pada permukaan kulit (flavido) buah *C. grandis* menjadikannya tidak menarik untuk dieksploitasi secara komersil. Laluan biosintesis karotenoid telah dikaji dengan mendalam dan dikenalpasti sebagai laluan biosintesis utama yang menyumbang kepada pembentukan warna flavido pada kebanyakan buah limau. Walaupun banyak informasi berkenaan biosintesis karotenoid di dalam flavido buah-buahan limau yang berwarna oren dan kuning, tiada informasi mengenai flavido buah berwarna hijau. Untuk meningkatkan permintaan pasaran di dalam dan di luar negara, adalah perlu untuk mengubah warna flavido *C. grandis* daripada warna hijau kekuningan yang pucat kepada warna yang lebih menarik sebagaimana warna buah limau yang lain. Maka, penyelidikan dimulakan untuk mengkaji kemungkinan mengubah warna flavido buah tersebut melalui penggunaan teknik-teknik molekul. Justeru itu, adalah penting untuk memencilkan dan membuat

analisis jujukan terhadap gen-gen yang terlibat dalam laluan karotenoid di dalam flavido hijau *C. grandis*. Analisis perbandingan jujukan yang diperolehi daripada gen-gen flavedo berwarna hijau tersebut akan dibandingkan dengan limau berwarna oren, tumbuhan lain dan bakteria yang mempunyai kandungan karotenoid yang tinggi. Kajian ini bermula dengan menanda bunga yang tersenyawa yang telah menunjukkan tanda pembentukan buah untuk memastikan peringkat kematangan yang betul, diikuti dengan pemencilan DNA dan RNA total sebelum analisis molekul seterusnya seperti PCR dan RTPCR. Primer yang digunakan di dalam teknik tersebut direkabentuk berdasarkan maklumat jujukan yang diperolehi daripada pengkalan data GenBank. Empat gen yang terlibat di dalam biosintesis karotenoid dan bertanggungjawab terhadap pembentukan pigmen oren pada buah limau iaitu; *fitoene sintase* (*psy*, 0.46 kb), *fitoene disaturase* (*pds*, 1.11 kb), *likopene β -siklase* (*lyc*, 0.70 kb) dan *β -karotene hidroksilase* (*chx*, 0.23 kb), telah dipencilkan melalui kaedah PCR dan RTPCR. Daripada analisis jujukan, *psy*, *pds*, *lyc* dan *chx*, persamaan yang tinggi dengan variati limau, *Prunus armeniaca* dan *Capsicum annuum*. Satu jujukan lengkap gen *chx* telah dipencilkan daripada kulit buah *C. grandis*. Jujukan lengkap 1268 bp gen *chx* tersebut mengandungi 271 kodon bacaan terbuka (ORF) (jisim 65.8 kD) dan 170-nucleotida 5' jujukan yang tidak ditranslasikan telah dipencilkan. Dengan membuat perbandingan terhadap organisma lain, tujuh residu histidin yang terdapat di dalam gen *chx* *C. grandis* juga ditemui dalam gen yang sama pada organisma *Alcaligenes sp* (no. akses D58422), *Agrobacterium aurianticum* (no. akses D58420), *Erwinia herbicola* (no. akses M87280) dan *Pseudomonas putida* (no. akses KT2440 NP_745389). Bahagian 'Motif 1' dan 'Predicted TM helix' juga terdapat di dalam *lyc- β* *C. grandis* apabila dibandingkan dengan *Prochlorococcus marinus* MED4 *CrtL- ϵ* (no. akses

CAE19092), *Lycopersicon esculentum* *Lcy-ε* (no. akses O65837), *Tagetes erecta* *Lcy-ε* (no. akses AAG10428), *Capsicum annuum* *Lcy-β* (no. laluan Q43415) dan *Arabidopsis thaliana* *Lcy-β* (no. akses AAA81880). Kesimpulannya, *psy*, *pds*, *lyc* dan jujukan lengkap *chx* telah berjaya dipencilkan daripada flavido *C. grandis*. Persamaan jujukan *psy*, *pds*, *lyc* dan *chx* adalah sangat tinggi dengan gen-gen yang berpadanan pada tumbuhan lain terutama sekali variasi limau.

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I certify that an Examination Committee has met on 28th January 2008 to conduct the final examination of Ummi Kalthum Hanapi on her degree in Master of Science thesis entitled “Isolation and Sequencing Analyses of cDNAs Involved in Carotenoid Biosynthesis in the Flavedo Tissue of Pummelo (*Cirus grandis* (L.) Osbeck)” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Doctor of Philosophy.

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DECLARATION

I declare that the thesis is my original work except for equations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at UPM or at any other institutions.

UMMI KALTHUM BINTI HANAPI

Date: 13/5/2008

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LIST OF ABBREVIATIONS

α	-	alpha
β	-	beta
ε	-	epsilon
γ	-	gamma
λ	-	lambda
μ	-	micro
ξ	-	zeta
ABA	-	abscisic acid
BLAST	-	Basic Local Alignment Search Tool
bp	-	basepair
BSA	-	bovine serum albumin
β CHX	-	β -carotene hydroxylase
ε CHX	-	ε -carotene hydroxylase
CCD	-	carotenoid cleavage dioxygenase
cDNA	-	complementary deoxyribonucleic acid
Ci	-	Curie
CoA	-	coenzyme A
C-terminal	-	carboxyl terminal
CTRB	-	bacterial phytoene synthase
<i>CrtB</i>	-	bacterial phytoene synthase gene
<i>CrtE</i>	-	bacterial gene encodes for enzyme responsible for the conversion of DMAPP to GGPP
<i>CrtI</i>	-	bacterial <i>carotenoid desaturase</i> gene

<i>CrtL-b</i>	-	<i>Cyanobacterium synechococcus</i> PCC 7942 lycopene ε -cyclase gene
<i>CrtL-e</i>	-	tomato lycopene β -cyclase gene
CRTISO	-	carotenoid isomerase
<i>CrtN</i>	-	bacterial carotenoid desaturase gene
CRTY	-	bacterial lycopene β -cyclase
<i>CrtY</i>	-	bacterial lycopene β -cyclase gene
DEPC	-	diethyl pyrocarboate
dH ₂ O	-	distilled water
DMAPP	-	dimethylallyl pyrophosphate
DMSO	-	dimethyl sulphoxide
DNA	-	deoxyribonucleic acid
DNase	-	nuclease
dNTPs	-	deoxynucleosides triphosphate
DOPA	-	L-3,4-dihydroxyphenylalanine
DXP	-	deoxyxylulose 5-phosphate
DXR	-	deoxyxylulose-5-phosphate reductoisomerase
DXS	-	deoxyxylulose-5-phosphate synthase
EDTA	-	ethylene diamine tetraacetate
EtBr	-	ethidium bromide
FAD	-	Flavin adenine dinucleotide
GA	-	gibberellin acid
GGPP	-	geranylgeranyl pyrophosphate
HCl	-	hydrochloric acid
IPP	-	isopentenyl pyrophosphate
kb	-	kilobase

kD	-	kilodalton
LB	-	Luria-Bertani
LDL	-	low density lipoprotein
LiCl	-	Lithium chloride
LCY β	-	lycopene β -cyclase
LYC ϵ	-	lycopene ϵ -cyclase
NCBI	-	National Center for Biotechnology Information
NTES	-	Natrium chloride-Tris-EDTA-SDS
MCS	-	multiple cloning site
MEP	-	2-C-methyl-D-erythritol 4-phosphate
MgCl ₂	-	magnesium chloride
MgSO ₄	-	magnesium sulphate
mRNA	-	messenger RNA
MVA	-	mevalonic acid
NaCl	-	natrium chloride
NAD	-	nicotinamide adenine dinucleotide
NADP	-	nicotinamide adenine dinucleotide phosphate
NaOAc	-	sodium acetate
NaOH	-	Natrium hydroxide
NCBI	-	National Centre for Biotechnology Information
<i>nced</i>	-	9- <i>cis</i> -epoxy-carotenoid dioxygenase gene
NSY or NXS	-	neoxanthin synthase
N-terminal	-	amino-terminal
OD	-	optical density
PCR	-	Polymerase Chain Reaction

PCI	-	phenol:chloroform:isoamyl alcohol [25:24:1 (v/v)]
PDS	-	phytoene desaturase
pfu	-	plaque forming unit
PPPP	-	prephytoene pyrophosphate
poly A ⁺ RNA	-	polyadenylated RNA
PSY	-	phytoene synthase
RNA	-	ribonucleic acid
mRNA	-	messenger RNA
rRNA	-	ribosomal ribonucleic acid
RNase	-	ribonuclease
ROS	-	reactive oxygen species
rpm	-	revolution per minute
RTPCR	-	Reverse Transcriptase Polymerase Chain Reaction
SA-PMPs	-	Streptavidin-Paramagnetic Particles
SDS	-	sodium dodecyl sulphate
SDS-PAGE	-	SDS-polyacrylamide gel electrophoresis
TE	-	tris-EDTA
TLC	-	thin layer chromatography
TM	-	transmembrane
Tris	-	tris[hydroxymethyl]amino methane
Tris-HCl	-	tris hydrochloride
U	-	unit
UV	-	ultraviolet
VDE	-	violaxanthin de-epoxidase
v/v	-	volume per volume

w/v	-	weigh per minute
x	-	times
xg	-	times gravity force
ZDS	-	ζ-carotene desaturase
<i>Zds</i>	-	ζ-carotene desaturase gene